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Lectin Magnetic Bead Array for Biomarker Discovery

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Abstract: Alterations in protein glycosylation play an important role in patho-physiology, and much effort has been devoted to detecting glycoprotein biomarkers. In this manuscript, we describe the development of a novel method for monitoring alterations in protein glycosylation. Lectins are used as individual affinity reagents and coupled to magnetic beads (Dynabeads) in a microplate array format for isolation of glycosylated proteins. Isolated glycoproteins are digested with trypsin in-solution followed by LC−MS/MS, allowing a liquid handler-assisted high throughput workflow. We demonstrate the specific and reproducible affinity-isolation of glycoproteins using the lectin Dynabead array technology. When used with serum, we achieved one-step purification of glycoproteins with minimal coisolation of abundant serum proteins including albumin. We further optimized the proteomics workflow to allow transfer to a liquid handler for automation. In summary, we report the development of a high throughput platform to detect alterations in protein glycosylation which will be useful in glycoproteomics studies, particularly clinical proteomics studies where large sample sizes are required to achieve statistical power.

Keywords: biomarker discovery • serum proteomics • glycosylation • cancer • lectin • magnetic beads

Introduction

The study of glycans (glycomics) and glycosylated proteins (glycoproteomics) has become a leading area of biomarker discovery activities in recent years.[1−5] Lectins, naturally occurring proteins that preferentially bind specific glycan moieties, are well-used research tools in glycoprotein studies. As early as the 1980s, lectin-coupled beads were used to capture glycoproteins.[6] Lectin immunohistochemistry was used to demonstrate altered glycosylation in breast cancer,[7] and more recently, lectin-affinity chromatography has been used in combination with mass spectrometry to examine the glycoproteome of pancreatic, breast, liver and colorectal cancer using serum, plasma or cells as sample source.[8−12] These studies demonstrate the utility and feasibility of lectin-affinity isolation-coupled proteomics as a means of identifying glycoproteins. These studies have used relatively small sample sizes, partly due to the high level of sample handling. To achieve statistical power for clinical proteomics studies, and to distinguish between different states of glycosylation, we set out to establish a high throughput platform using lectins as monoaffinity reagents. In this Technical Note, we report the development a proteomics workflow using lectin-coupled magnetic beads, in-solution digest and LC−MS/MS for isolation and identification of serum glycoproteins. This workflow achieved a one-step purification of serum glycoproteins, removing the need for depletion, separation or cleanup steps. Importantly, we show that protein binding to lectin-magnetic beads is lectin-selective and highly reproducible.

Experimental Section

Reagents. MyOne and M280 magnetic Dynabeads were from Life Technologies (Dynal). Lectins and lectin-coupled agarose beads were purchased from Vector Laboratories. Modified sequencing grade bovine trypsin was from Promega. All other reagents were from Sigma unless otherwise specified.

Patient Sample. Blood samples were collected with consent and ethics approval by the Study of Digestive Health (Queensland Institute for Medical Research) and accessed with approval by University of Queensland Human Ethics Committee. Sera were stored in aliquots at −80 °C.

Preparation and Use of Lectin-Dynabeads. Lectin coupling to surface-activated Dynabeads was performed according to manufacturer's protocol, and the beads were then blocked with 1 M Tris buffer pH 7.5 for 16 hours. Lectin-Dynabeads were washed 3 times with binding buffer containing 20 mM Tris/HCl pH 7.4, 15 mM NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, (1% Triton X-100, 0.2% SDS, 1 mM DTT where indicated) and protease inhibitors (1 μg/μL Aprotinin, 1 μg/μL Antipain, 1 μg/μL Pepstatin, 1 μg/μL Leupeptin and 2.5 mM Benzamidine). Protein sample (2 μL of serum) was incubated with lectin-coupled beads diluted in binding buffer as specified and rotated at 4 °C for 30 min. After glycoprotein capture, beads were washed three times with 10 volumes of binding buffer. Protein elution methods depended on subsequent analysis. For 2D gel electrophoresis, proteins were eluted with 2D sample buffer (7 M urea, 2 M thiourea, 4% CHAPS, 100 mM DTT 0.5% ampholyte 3−10, trace bromophenol blue) for 30 min at room temperature prior to rehydrating pH 3−11, nonlinear, 7 cm Immobilzed pH gradient strips (GE healthcare). Isoelectric point focusing was performed using the OffGel Fractionator (Agilent) programmed for total of 8 kVh, limited at 5000 V, 50 μA, 200mW and 5 h. After equilibration with 2% DTT and then 2.5% IAA in equilibration buffer (6 M urea, 375 mM Tris/HCl pH 8.8, 2% SDS, 20% glycerol), strips were placed on top of

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10% mini-SDS-PAGE gels and subjected to electrophoresis. Gels were stained with colloidal coomassie.[13] In-gel digest was performed as previously described.[14]

For direct in-solution digest and LC−MS/MS, beads were additionally washed twice with 200 mM Tris/HCl pH 7.4, twice with 50 mM NH_4HCO_3, then the proteins were eluted with one volume of 50% formic acid followed by one volume of 20% acetonitrile. The formic acid in the eluate was neutralized with two volumes of 50 mM NH_4HCO_3. The samples were dried in a speedy vac, resuspended in 20 μL of NH_4HCO_3 and digested with 0.5 μg trypsin (Promega sequencing grade modified bovine trypsin) overnight at 37 °C. Trypsin was inactivated by acidification to 5% formic acid. Samples were dried in a speedy vac and resuspended in 40 μL of 5% formic acid.

LC−MS/MS. Tryptic peptides corresponding to 5% of lectin-Dynabead pulldown (approximately 1 μg of protein from 2 μL of starting serum) were subjected to LC−MS/MS using an Agilent 6520 QTOF coupled with a Chip CUBE and 1200 HPLC. The nano pump was set at 0.3 μL/min and the capillary pump at 4 μL/min. The HPLC-chip used contained a 40nl C18 trapping column, and a 150 mm C18 resolving column (Agilent). Buffer A was 0.1% formic acid and buffer B was 0.1% formic acid in 90% acetonitrile. For in-solution digest, the gradient went from 10% to 50% Buffer B in 45 min, and then 95% Buffer B at 46 min, returning to 10% buffer B at 50 min. For 2D gel spots, the gradient went from 10% to 50% Buffer B in 20 min, and then 95% Buffer B at 20.10 min, returning to 10% buffer B at 22.10 min. The mass spectrometer was programmed to acquire 8 precursor MS1 spectra per minute and 4 MS/MS spectra per minute. Dynamic exclusion was applied after 2 MS/MS within 0.25 min. Results were searched against IPI human v3.64 database using Spectrum Mill (Agilent A03.03). The following parameters were used for search: 2 maximum missed cleavage, precursor mass tolerance of ±20 and product mass tolerance of ±50. For 2D spots and when testing reduction/alkylation in solution, carbamidomethylation was selected as fixed modification.

Safety Considerations. Some lectins can be mitogenic or cytotoxic, material safety datasheet should be consulted for each lectin and appropriate personnel protective equipment used.

Results and Discussion

In order to achieve sample throughput for clinical proteomics studies, we wished to establish a high throughput automatable procedure that links protein isolation/separation to mass spectrometry. Magnetic beads are easily adaptable to robotic liquid handlers and thus represent the support of choice when considering high throughput affinity isolation procedures. Since our affinity reagents, lectins, are proteins, we examined the functionalized Dynabeads that were recommended for direct protein coupling, namely epoxy, tosyl and carboxylic acid activated Dynabeads (LifeTechnologies, Dynal). Preliminary results suggest that epoxy and tosyl-activated beads are better than carboxylic beads at capturing wheat germ agglutinin (data not shown), therefore we further compared the coupling efficiency of tosyl and epoxy activated beads with commercial agarose beads (Vector Laboratories) for Concanavalin A (ConA), wheat germ agglutinin (WGA) and Jacalin (JAC), with visualization by colloidal Coomassie staining. Tosyl-activated beads captured the highest level of lectin, as judged by coomassie staining (Figure 1). We selected 1 μm diameter MyOne tosyl-activated beads (Life Technologies Dynal) for use in further

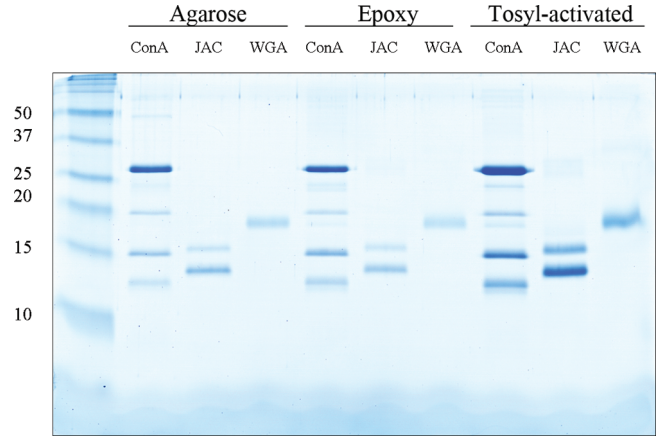

Figure 1. Comparison of binding efficiency of Concanavalin A (ConA), Jacalin (JAC) and wheat germ agglutinin (WGA) to agarose and Dynabeads. Each lectin was bound to epoxy and tosyl activated magnetic beads to the same concentration as commercial agarose beads (ConA 6 mg/mL, Jac 4 mg/mL, WGA 7 mg/mL). Lectins were eluted from 2 μL of beads and examined on 17% SDS-PAGE and colloidal coomassie staining.

experiments because the small diameter of the beads offers more surface area for protein capture.

Initial experiments comparing the ability of lectin magnetic beads to isolate glycoproteins from serum with commercial lectin agarose beads showed comparable results (data not shown), suggesting that lectin magnetic beads will be suitable for glycoprotein isolation. One important consideration in lectin pull down experiments is the possibility of isolating protein complexes, leading to the erranous assignment of nonglycosylated proteins, or glycoprotein with other glycan moieties. Thus the use of binding buffer should be carefully considered to reduce the presence of protein complexes, while retaining lectin-glycan interactions. To this end, we compared pull down using ConA-tosyl-activated Dynabeads under four different binding buffers. Condition 1 used a lectin binding buffer from Yang and Hancock[15] that contained 20 mM Tris/HCl pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, with additional 0.05% Triton X-100, and protease inhibitors as described in Experimental Section. This represents a mild buffer condition with minimal salt and detergent. This buffer was used during binding and washing steps. In condition 2, we included 0.5 M urea as denaturing agent in the base buffer in the binding and washing steps to disrupt protein complexes. In condition 3, additional 1 mM DTT, 0.2% SDS, and 1% Triton-X100 was included in the wash buffer to increase the stringency of the washing step. In the most stringent condition, the additional DTT, SDS and Triton-X100 was included in both the binding and wash buffers (condition 4). Since this condition contained reducing agent and strong detergent during the entire pull down, it was possible that lectin binding efficiency will be reduced. After washing in binding buffer, bound proteins were eluted in 2D sample buffer and analyzed by 2D gel electrophoresis with 2D standards loaded with each sample (Figure 2). Compared to input serum, the ConA-Dynabead pulldown showed no visible albumin spot, demonstrating the selectivity of the pulldown. Similar number of spots was observed in all four conditions, with the numbers being 60, 57, 46, 50 spots for conditions 1−4, respectively. While the inclusion of reducing agent and strong detergent in binding and washing steps (condition 4) resulted in ~20% loss of protein binding, we still

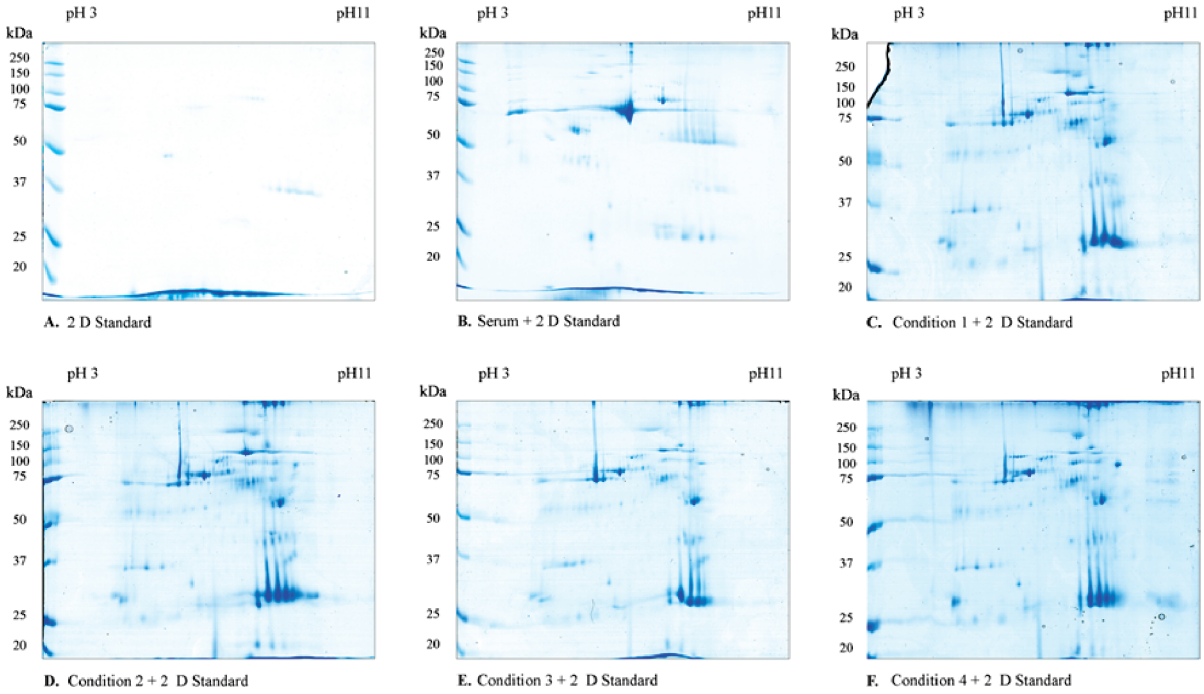

Figure 2. Pulldown of serum proteins using ConA-Dynabeads.Glycoproteins were isolated from control patient serum (2 mL) using Con A-coupled tosyl-activated magnetic beads using four different buffer conditions from mild (condition 1) to most stringent (condition 4) as described in Results and Discussion. Bound proteins were eluted in 2D sample buffer and analyzed by 2D electrophoresis and colloidal coomassie staining, together with 2D standard. A 0.2 μL aliquot of the input serum is analyzed in (B) for comparison.

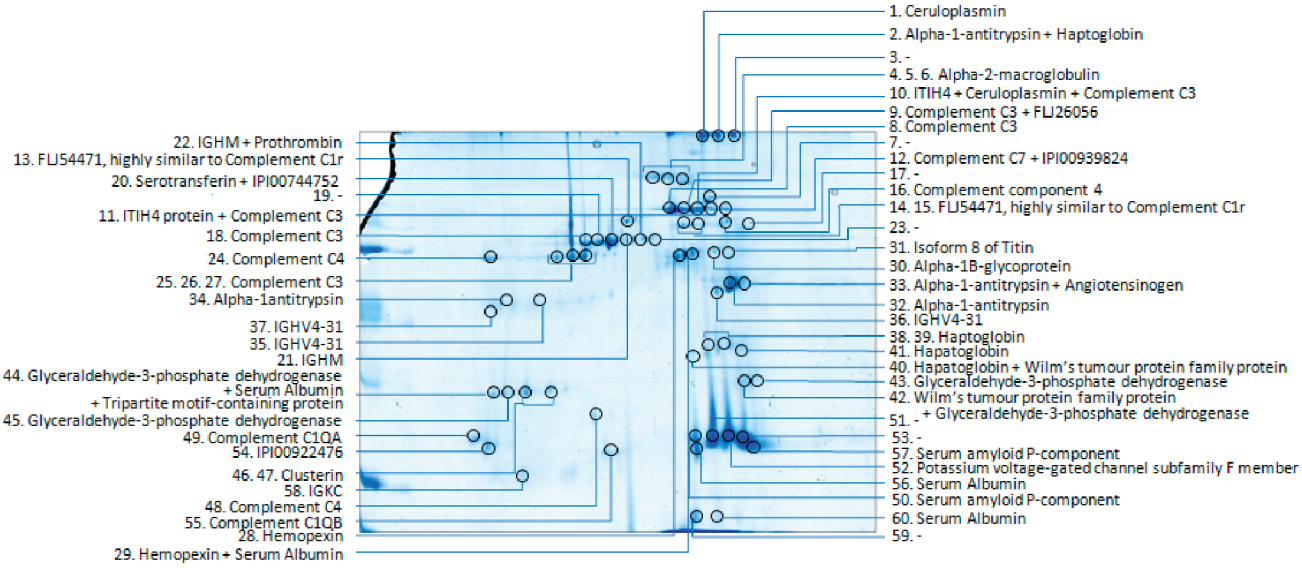

Figure 3. LC−MS/MS identification of ConA-Dynabead-binding serum proteins. 2D gel spots were excised, digested with trypsin and subjected to LC−MS/MS analysis as detailed in Experimental Section.

observed close to 50 spots, demonstrating the strong affinity between lectin and their cognate glycans.

Sixty spots were excised from gel 1, subjected to in-gel tryptic digest and LC−MS/MS. Fifty-two spots were positively identified (Figure 3, protein identification table available as Supplementary Table 1, Supporting Information), with several proteins identified in multiple spots giving a final of 31 proteins. Eight spots were unidentified, either due to low abundance or the intrinsic interference of highly modified peptides by LC−MS/MS identification. The latter cause may be resolved by glycosidase treatment of samples. Out of the 31 ConA-isolated proteins, 18 proteins were annotated in UniProt as glycosylated (Supplementary Table 1, Supporting Information). Several of the other 13 ConA-isolated proteins were uncharacterized sequences, while some were likely cobinding proteins such as albumin. Further work using these beads may lead to identification of novel glycosylation sites. The most stringent condition, condition 4 was used in later experiments to prevent isolation of nonglycoproteins using the ConA magnetic beads.

To develop a high throughput workflow, we wished to perform in-solution tryptic digest of the isolated proteins, and to directly inject sample to LC−MS/MS without clean up. To this end, we specifically investigated two steps in the workflow. First, we compared reduction/alkylation and clean up of the

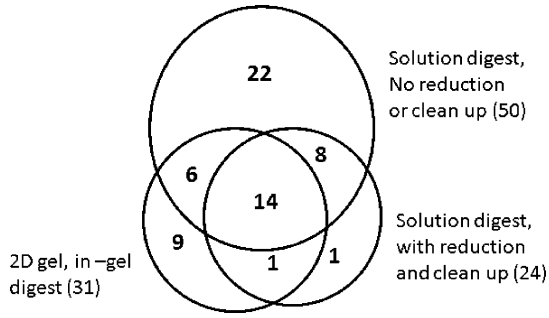

Figure 4. Number of proteins identified from ConA-Dynabeads pulldown from serum. Overlap between serum proteins isolated using ConA-Dynabeads were subjected to in-solution digest with or without reduction, alkylation and STAGE-tip clean up, or analyzed by 2D gel and in-gel digest of spots.

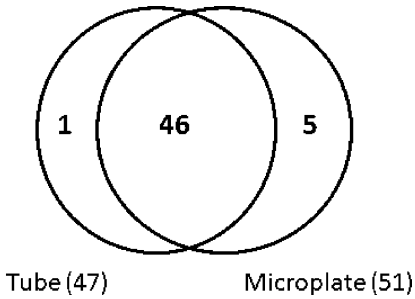

Figure 5. Comparison of tube versus microplate performace for lectin-Dynabead pulldown coupled to in-solution digest and LC–MS/MS.

in-solution digest (the "standard" nonhigh throughput workflow) with a shortened protocol that leaves out these steps. After washing the pulldown in binding buffer, and further washing in 50 mM NH_4HCO_3, bound proteins were sequentially eluted with 50% formic acid and 20% acetonitrile. After drying in a speedvac, the samples were divided into two workflows. For one set, proteins were reduced, alkylated, digested with trypsin and then cleaned up with a C18 STAGE-tip.[16] For the second set, proteins were directly digested without reduction alkylation. This experiment was designed to test if a shortened in-solution digest procedure without reduction/alkylation produces acceptable number of protein identifications. Peptides were analyzed by LC–MS/MS and database searching. Three technical replicates were performed. A protein identity was considered safe if it was identified in 2 out of the 3 replicates (Supplementary Table 2, Supporting Information). Based on these criteria, 50 proteins were identified from ConA-Dynabead pulldowns using in-solution digest without reduction/alkylation and clean up, in contrast to only 24 identified when reduction/alkylation and STAGE-tip clean up was performed (Figure 4, Supplementary Table 2, Supporting Information). The reduced number of protein identified in the second protocol may be due to significant sample loss during the clean up step. Analysis by of similar pulldown 2D gel and in-gel digest had identified 31 proteins (Figure 3, Supplementary Table 1, Supporting Information). When the overlap between the 3 protein identification procedures were compared, we found that 14 proteins were identified by all 3 methods, while 22 proteins were identified only using solution digest without reduction/alkylation. The 2D gel protocol identified 9 proteins that were not found using solution digest. However, the entire pulldown was loaded onto a single 2D gel, while only 5% of the sample was loaded on the LC–MS/MS from the solution digest procedures, suggesting a much reduced rate of protein identification with 2D gel separation. Based on these results, we selected in-solution digest without reduction/alkylation as the protein processing method prior to LC–MS/MS.

As our aim is to develop a liquid handler-assisted high throughput workflow, we next examined if the entire procedure performs as efficiently in 96 well microplates. For this experiment, we divided ConA-Dynabeads into 6 aliquots, one set in 3 separate eppendorf tubes and one set in 3 wells of a 96 well microplate. Pulldown was performed from aliquots of a single serum sample, using a hand-held 96 well microplate bar magnet (Life Technologies) for the microplate samples. All samples were processed in parallel using in-solution digest without reduction/alkylation, and analyzed by LC–MS/MS. Protein identities were confirmed if 2 out of the 3 technical replicates identified the protein. Surprisingly, we found that performing the procedure in 96 well microplates actually identified slightly more proteins, and the scores for protein identification were also better with microplates (Figure 5, Supplementary Table 3, Supporting Information). The majority proteins were identified in both vessel formats, supporting the consistency of the lectin-Dynabead pulldown workflow. We analyzed the reproducibility between the 3 technical replicates for the tube and microplate workflows. Protein identities were highly reproducible in both microplate and tube workflows, with the majority of proteins identified in all 3 technical replicates (Figure 6, Supplemental Table 3, Supporting Information). These results suggest that transferring the lectin-Dynabead pulldown workflow to microplates will not reduce efficiency but may in fact enhance the sensitivity and confidence of protein identifications.

Table 1. Selectivity of Lectin Magnetic Bead Pulldown[a]

known target		ConA	Jac	SBA	SNA	STA	UEA I	WGA
α-Man, α-Glc, and α-GlcNAc	ConA	53	16	5	38	19	8	20
Galα1–6GalNAc and Galβ1–3GalNAc	Jac		49	6	17	8	5	9
GalNAcα1–3Gal	SBA			8	4	5	10	4
Neu5Acα2–6Gal and Neu5Acα2–6GalNAc	SNA				53	22	10	20
GlcNAcβ1–4GlcNAc oligomers	STA					36	10	21
Fucα1–2Galβ1–4GlcNAc	UEA I						13	9
Neu5Ac and GlcNAcβ1–4GlcNAc	WGA							31

[a] An array of seven lectin-coupled tosyl-activated beads was prepared and used to isolate serum glycoproteins from a single patient serum. Proteins were eluted, digested in-solution and analysed by LC–MS/MS. The table shows the known glycan target of each lectin, and the number of overlapping proteins identified between pairs of lectin pulldowns. Protein identification table is available as Supplementary Table 4 (Supporting Information). Abbreviations: Fuc, fucose; Gal, galactose; GalNAc, *N*-Acetylgalactosamine; Glc, glucose; GlcNAc, *N*-Acetylglucosamine; Man, mannose; Neu5Ac, *N*-acetylneuraminic acid (also called sialic acid); ConA, concanavalin A; Jac, Jacalin; SBA, Soy Bean Agglutinin (Glycine max lectin); SNA, Sambucus Nigra Agglutinin; STA, Solanum Tuberosum Agglutinin; UEA I, Ulex Europaeus agglutinin I; WGA, wheat Germ agglutinin.

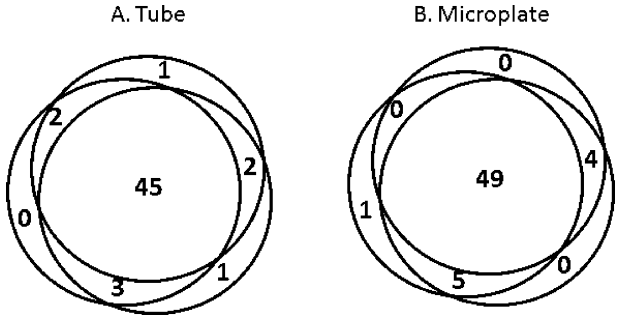

Figure 6. Reproducibility of lectin Dynabead pulldown. ConA-coupled magnetic beads was used to isolate serum glycoproteins, which were eluted, digested in-solution and identified by LC−MS/MS. The Venn diagram shows the reproducibility of the procedure over 3 independent technical repeats when used with (A) Eppendorf tubes and (B) 96-well microplates.

To demonstrate the utility and selectivity of lectin-Dynabead array, we prepared a panel of 7 lectin-Dynabeads and used this small array in a pulldown experiment. The lectins were selected based on their reported glycan affinities, covering both N- and O-glycosylation as well as sialic acid modification (Table 1). When aliquots of a single serum sample were subjected to pulldown using this panel of lectin-Dynabeads, we observed selective binding of proteins to the lectin-Dynabeads (Table 1, Supplementary Table 4, Supporting Information). Most identified proteins bound to several lectins, reflecting the fact that glycoproteins are often modified with multiple types and sites of glycosylation. Table 1 shows the number of proteins isolated by each lectin, and the number that is identified in pulldown from pairs of lectin-Dynabeads. ConA and SNA (*Sambucus nigra* Agglutinin) bound the highest number of proteins, with more than 50% of the proteins in common. These may indicate that ConA and SNA have similar binding specificities, or the target glycans preferentially occur together. Glycomic analysis of the isolated proteins will be required to further characterize the glycan modifications. In contrast to ConA and SNA, which have broad target specificity, SBA (Soy Bean Agglutinin) has a narrow glycan selectivity, and only 8 proteins were isolated by SBA-Dynabeads (Table 1). Thus, by combining lectin-Dynabead array with bioinformatic analysis of the protein (and peptide/glycan) identifications, this workflow has the potential to identify alterations in glycosylation profiles due to disease.

Conclusions

We have developed a novel high throughput technique for biomarker discovery and glycoproteomics studies. This method is based on the use of lectin-Dynabeadsto isolate glycoproteins in a glycan-specific manner. The increased throughput means that many lectins can be tested per sample, and alterations in lectin binding ability can be determined for many biological samples. The utility of lectin magnetic beads as a one-step serum glycoprotein isolation procedure will greatly assist identification of serum biomarkers. Development of glycomic analysis workflows combined with bioinformatic and biostatistic analysis algorithms will be required to fully utilize this technology for biomarker discovery.

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Supporting Information Available: Supplementary Tables 1−4. This material is available free of charge via the Internet at http://pubs.acs.org.

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